Volume 14 Article 63

4-1-1987

Research Notes : United States : Screening of the USDA soybean cultivar collection for slow and fast urease isozymes

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1) Screening of the USDA soybean cultivar collection for slow and fast urease isozymes.

Urease present in soybean seed is known to consist of two isozyme variants. These variants are referred to as electrophoretically fast or slow on polyacrylamide gels and are believed to be polymers of a single subunit (Buttery and Buzzell, 1971; Polacco and Havir, 1979). The slow variant, considered a hexamer, has been estimated at 480,000 daltons by Polacco and Havir (1979) and at 520,000 daltons by Buttery and Buzzell (1971). Although data on the fast form of urease is limited, it is believed to be a trimer of approximately 205,000 daltons (Buttery and Buzzell, 1971). Kloth and Hymowitz (1985) have presented evidence that the urease isozymes are codominantly inherited. They assigned the gene symbol *Eul-a* to the slow-moving allele and *Eul-b* to the fast-moving allele.

Polacco (1985, personal communication) screened about 8500 soybean plant introductions for the presence or absence of urease activity. However, he did not determine which soybean cultivars contain the fast or slow urease variants. The objectives of this study were (1) to screen the USDA soybean cultivar collection (Canada and USA public releases) for urease isozymes; and (2) to determine if there are positive associations between type of electrophoretic banding pattern and maturity group, geographical origin or year of release.

The 397 soybean cultivars used in this study were obtained from R. L. Bernard, USDA, Urbana, Illinois. Seed extracts and seed urease isozymes were determined by the procedure of Kloth and Hymowitz (1985).

The distribution of seed urease variants in soybean cultivars by their year of introduction or release up to 1985 is summarized in Table 1. Analysis of 397 cultivars revealed nearly equal numbers of those containing slow and fast seed urease variants. The slow variant was about twice as prevalent as the fast variant in soybean cultivars released through 1939. Approximately 90% of the 31 soybean cultivars whose origin was Japan contained the slow urease allele.

The soybean cultivars from China (48) and Korea (16) were about equally divided between the slow and fast urease variants. The soybean cultivars released from 1940 through 1969 contained about an equal number of fast and slow seed urease variants. However, of the 110 cultivars released since 1970, 70% contain the fast variant of seed urease. Furthermore, 84% of the 44 cultivars released since 1980 contain the fast isozyme. Thus, an apparent reversal on the predominant form of seed urease has occurred over time, whereby the fast variant has become prevalent in the newly released soybean cultivars.

An analysis of recently released cultivars that contain the slow variant reveals that 15 of 18 released since 1976 contain 'Harosoy' or a derivation of Harosoy in their pedigree. Harosoy, a cross between 'Mandarin (Ottawa)' and 'A.K. (Harrow)' was released in 1951 (Hymowitz et al., 1977),

and its seed contains the urease slow variant. Thus, the majority of recently released public soybean cultivars containing the seed urease slow variant can be traced back to a narrow germplasm pool.

Table 2 contains the distribution of seed urease variants in 297 soybean cultivars by maturity group. No significant conclusions can be drawn from the data.

Table 1. Distribution of seed urease variants in soybean cultivars by year of introduction or release

Year	Urease form		Total
	Fast	Slow	
-1899	1	3	4
1900-1909	8	19	27
1910-1919	6	12	18
1920-1929	20	41	61
1930-1939	12	21	33
1940-1949	24	24	48
1950-1959	14	16	30
1960-1969	32	31	63
1970-1979	40	26	66
1980-	37	7	44
Total	194	203[a]	397[a]

[a]Date of release information not available for three cultivars containing the slow isozyme of urease.

Table 2. Distribution of seed urease variants in soybean cultivars by maturity group

Maturity group	Urease		Total
	Fast	Slow	
000	0	1	1
00	3	18	21
0	11	12	23
I	26	21	47
II	28	37	65
III	45	24	69
IV	42	31	73
V	8	9	17
VI	18	10	28
VII	9	14	23
VIII	3	25	28
IX	1	1	2
X	0	0	0
Total	194	203	397

Acknowledgment: Research supported in part by a Roney undergraduate research award to T.L.M.

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2) Screening of the USDA *Glycine soja* collection for urease variants.

Two isozyme variants of seed urease are present in soybeans. These variants are referred to as electrophoretically fast and slow on polyacrylamide gels (Buttery and Buzzell, 1971; Polacco and Havir, 1979). Kloth and Hymowitz (1985) demonstrated that the urease isozymes are codominantly inherited. They assigned the gene symbol *Eul-a* to the slow-moving allele and *Eul-b* to the fast-moving allele.

Of about 8500 soybean accessions screened for the presence or absence of seed urease activity (Polacco, 1985, personal communication), four accessions were identified having a seed urease null phenotype: PI 229324 ('Itachi') (Polacco et al., 1982) and PI 416975 ('Kairyou Kakushin'), PI 417073 ('Kounou 1'), and PI 417074 ('Kounou 2') (Kloth et al., 1987). The absence of urease activity apparently is controlled by a null lesion (*sun*) and is linked to *Eul* by approximately one map unit. In the presence of *Sun*, *Eul-a* or *Eul-b* is expressed in soybean seed (Kloth et al., 1987).

No screening of the *Glycine soja* Sieb. and Zucc. seed for urease isozyme variants has been done. *Glycine soja* is the wild annual ancestor of the soybean. The objectives of this study were (1) to screen the USDA *G. soja* collection for the slow and fast urease isozymes and (2) to determine if there were any null phenotypes within the collection.

The 629 *G. soja* accessions used in this study were obtained from R. L. Bernard, USDA, Urbana, Illinois. Seed extracts and seed urease isozymes were determined by the procedure of Kloth and Hymowitz (1985). Seed of *G. soja* lacking urease bands were checked for urease activity by the urease color test described by Kloth (1985).

Results of the *G. soja* screening for seed urease banding pattern are summarized in Table 1. Five accessions (PI 407074, 407117, 407118, 407119, 407120) lacked seed urease. These five accessions came from Japan. It is of interest to note that the four soybean accessions lacking seed urease also came from Japan. The rest of the 624 *G. soja* accessions analyzed contained the slow variant.

Table 1. Distribution of seed urease variants and urease nulls in the USDA *Glycine soja* germplasm collection

	Urease form			
Origin	Fast	Slow	Null	Total
China	0	97	0	97
Japan	0	177	5	182
Korea	0	314	0	314
Taiwan	0	2	0	2
USSR	0	34	0	34
Total	0	624	5	629

The slow urease band in *G. soja* (PI 391.587) migrated to the same position as soybean cultivar 'Columbia', the slow urease band standard. In addition, an artificial hybrid (mixture of seed extracts) containing both PI 391.587 and Columbia formed a single slow urease band.

From these data, it appears as though Japan is the geographical origin of seed lacking urease. The null phenotype only was found in *G. max* and *G. soja* seed from that country. The absence of the fast urease variant in the 629 *G. soja* accessions screened is intriguing. Perhaps the fast urease variant was derived as a mutational event in the domesticated soybean.

Acknowledgment: Research supported in part by a Roney undergraduate research award to T.L.M.

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3) Isozymes as predictors of ploidy level in *Glycine tabacina* (Labill.) Benth.

Glycine tabacina (Labill.) Benth. and *G. tomentella* Hayata have both diploid (2n=2x=40) and tetraploid (2n=4x=80) forms (Singh and Hymowitz, 1985a). Although the diploid plants can be distinguished cytologically from the tetraploids, the procedure is tedious, and requires precious greenhouse space.

Seed isozyme analysis may prove to be a simpler approach for differentiating between the diploid and tetraploid tabacinas. Grant et al. (1984) have demonstrated that zymogram patterns were predictive of ploidy level in *G. tomentella*. Specifically, they reported that shikimate dehydrogenase and endopeptidase were good predictors of ploidy level. However, the isozymes could not distinguish between the 38 vs. 40 or the 78 vs. 80 *G. tomentella* cytotypes.

The objective of this investigation was to identify the ploidy level of *G. tabacina* accessions of unknown chromosome number in order to facilitate the organization of the germplasm collection for further cytological and genetic studies. Thirteen recently introduced *G. tabacina* accessions from Australia were used in the study. Two known accessions, a diploid tabacina (PI 339.661) and a tetraploid tabacina (PI 193.232) were used as checks. Five dry seed per accession were used in the analysis. The extraction, starch gel electrophoresis and staining procedures were carried out according to the procedure outlined by Menancio and Hymowitz (n.d.). The enzymes used were: (1) alcohol dehydrogenase (ADH); (2) malate dehydrogenase (MDH); (3) phosphoglucoisomerase (PGI); and (4) shikimate dehydrogenase (SDH). The choice of enzymes was based upon screening data obtained from 68 *G. tabacina* accessions of known chromosome number.

Three out of the four enzyme systems used were able to differentiate between the (6) diploid and (7) tetraploid tabacina accessions of unknown chromosome number. These were MDH, PGI and SDH. ADH yielded single bands for diploids but certain tetraploids exhibited this same pattern. The disappearance of an MDH band in the multibanded region of the MDH pattern (see Broué et al., 1977) was found to be diagnostic of diploid accessions. Similarly, the SDH patterns described by Grant et al. (1984) for *G. tomentella* were likewise observed among the diploid vs. tetraploid tabacina accessions. The greater number of bands and more complicated patterns of PGI were to a certain degree predictive of the tetraploid form. The increased number of bands observed in the tetraploid tabacinas suggest that these forms probably contain more than one genome. These findings are concordant with the cytogenetic evidence of Singh and Hymowitz (1985b) that tetraploid *G. tabacina* is an allopolyploid complex.

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4) Examination of wild perennial *Glycine* species for glyphosate tolerance.

There has been much recent interest in the development of crops that are tolerant or resistant to the herbicide glyphosate. Glyphosate is a nonselective systemic herbicide that can control many annual and perennial weeds regardless of their size, a characteristic atypical of most selective herbicides used in crop production. Crop tolerance to glyphosate would be beneficial as it would provide growers, for the first time, the opportunity to control almost all weeds with a single herbicide application. The wild progenitors of cultivated crops have in the past provided favorable genetic traits that often can be incorporated into these crops. We have been screening accessions of wild perennial *Glycine* species for tolerance to glyphosate. The existence of tolerance in wild germplasm could lead to the development of a glyphosate-tolerant soybean cultivar.

Materials and methods:

Greenhouse Studies were conducted to evaluate 173 accessions of the following seven *Glycine* species for glyphosate tolerance: *G. canescens*, *G. clandestina*, *G. cyrtoloba*, *G. falcata*, *G. latifolia*, *G. tabacina*, and *G. tomentella*. Seeds were nicked with a razor blade for scarification, and germinated on moist filter paper at 25 C until radicles emerged. Germinated seeds were planted in plastic tubes with a volume of 175 cm^3 containing a growth medium of two parts soil, one part vermiculite, and one part sand. These species grow slowly for an extended period following emergence, after which they begin to grow more rapidly. Glyphosate was applied to plants when they reached the rapid growth stage, since systemic herbicides are most effective when plants are most actively growing.

Glyphosate rates of 0.28 to 0.56 kg/hectare (kg/ha) are sufficient to kill soybeans, while rates of at least 0.56 kg/ha are required for weed control. In initial studies, glyphosate was applied to plants at a rate of 0.28 kg/ha and plants visually rated for injury two weeks following application. Surviving plants were retreated with 0.56 kg/ha and rated as before. Glyphosate application continued in a sequential manner in 0.28 kg/ha increments to a rate of 1.4 kg/ha. Accessions tolerant to 1.4 kg/ha were replanted and treated with a 2.2 kg/ha rate of glyphosate. Accessions surviving 2.2 kg/ha were replanted and treated with 1.1 and 2.2 kg/ha of glyphosate to confirm previous observations. *Glycine max*, 'Williams 82', was included in the latter study as a comparison.

Cell culture. Experimental materials included *G. max* 'Asgrow 3127', *G. tabacina* and *G. cyrtoloba*. *Glycine tabacina* and *G. cyrtoloba* seeds were surface-sterilized by passage through 30% commercial bleach (5.25% sodium hypochlorite) for 30 minutes and rinsed three times in sterile distilled water. Seeds were germinated aseptically on agar-solidified nutrient medium. Approximately 10-day-old seedlings were used to provide 1-cm hypocotyl pieces for inoculation onto modified PC-L2 agar medium described by Phillips and Collins for callus production. Adjustments to the PC-L2 were addition of 2,4-D, NAA and kinetin at 0.4, 4.7 and 2.2 mg/liter, respectively, and elimination of picloram. Two- to four-month-old callus was used to initiate cell suspension cultures by inoculating 1.0 to 1.5 g tissue into ca. 10 ml of callus initiation media except that the 2,4-D concentration was increased to 1.2 mg/l. Cell suspensions were subcultured weekly by using 5 ml of suspension to inoculate 40 ml of fresh media in 125 ml Erlenmeyer flasks. After one week, cell densities averaged 0.3 g fresh wt/ml. Cultures were rotated at 130 rpm under continuous indirect fluorescent light at 27 C. A two-year-old cell suspension of *G. max* was obtained from Dr. Jack Widholm (University of Illinois) and maintained under conditions described above in PC-L2 with 2,4-D at 0.4 mg/l and no picloram. Glyphosate treatments were prepared by adding 2.5 ml glyphosate stock to 40 ml of fresh media prior to subculture. Glyphosate stocks contained 4.8 g MES/l and pH adjusted to 5.8. Cell fresh weights were obtained one week after treatment.

Results and discussion:

In greenhouse studies, differential response of accessions to glyphosate was observed among and within species. All species contained at least one accession able to survive a glyphosate rate of 1.4 kg/ha (Table 1). Differences among species became more apparent, however, upon consideration of the relative injury sustained by accessions in response to this glyphosate rate. Accessions of *G. clandestina*, *G. cyrtoloba*, and *G. tabacina* were the only plants to survive with less than 20% injury. Some accessions of *G. falcata* and *G. tomentella* survived with between 20% and 40% injury, along with additional accessions of the previous three species. Accessions of *G. canescens* and *G. latifolia* were least tolerant to glyphosate, since none survived 1.4 kg/ha glyphosate with less than 40% injury.

Treatment of the most tolerant accessions with 2.2 kg/ha of glyphosate resulted in the survival of plants from two *G. cyrtoloba* accessions and three *G. tabacina* accessions. When these five accessions were replanted and rescreened, all survived 1.1 kg/ha of glyphosate with 50% injury or less (Table 2). However, all plants except those in one *G. tabacina* accession were killed at the 2.2 kg/ha rate. While results in the last two studies were not entirely consistent, we feel tolerance was demonstrated, especially since all *G. max* plants were killed at 1.1 kg/ha of glyphosate.

Table 1. Response of *Glycine* spp. accessions to 1.4 kg/ha of glyphosate

Species	Number screened	Number surviving	<40% injury	<20% injury
G. tabacina	69	60	15	3
G. clandestina	35	20	7	2
G. tomentella	29	24	4	0
G. canescens	22	4	0	0
G. latifolia	11	3	0	0
G. cyrtoloba	5	3	1	1
G. falcata	2	1	1	0

Table 2. Response of five tolerant *Glycine* spp. accessions and *G. max* to 1.1 and 2.2 kg/ha of glyphosate

		% Injury	
Species	Accessions	1.1 kg/ha	2.0 kg/ha
G. tabacina	A	50	63
G. tabacina	B	45	100
G. tabacina	C	43	100
G. cyrtoloba	A	20	100
G. cyrtoloba	B	30	100
G. max	'Williams 82'	100	100

Table 3. Response of *Glycine* spp. cell suspension cultures to glyphosate

Glyphosate concentration (µmolar)	Fresh weight of cells (% of control)		
	G. cyrtoloba	*G. tabacina*	*G. max*
100	95	18	75
250	47	13	31
500	41	0	23
1000	25	0	0

Cell suspension cultures were initiated for one accession each of *G. tabacina* and *G. cyrtoloba*. These two accessions showed the greatest tolerance to glyphosate in greenhouse studies. Cell fresh weights of *G. cyrtoloba* were greater than those of *G. max* at all glyphosate concentrations (Table 3). However, differences in the fresh weights between *G. cyrtoloba* and *G. max* cells at the four concentrations ranged from only 16 to 25%. Differences of this magnitude may not be sufficient to attribute tolerance observed at the whole plant level entirely to biochemical factors, and indicate that differential uptake and translocation of herbicide from the leaf surface may be partially responsible for tolerance.

Glycine tabacina cells were less tolerant of glyphosate than those of *G. max*. This difference in response was inconsistent with greenhouse studies, where the order of tolerance was reversed. These data would tend to support a hypothesis that, for some accessions, tolerance differences observed at the whole plant level are due to uptake and translocation differences, rather than differences at the active site of the herbicide, the enzyme EPSP-synthase.

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5) Preliminary investigations on the salt tolerance of wild perennial *Glycine* species.

In 1983, 1984, and 1985, the senior author collected wild perennial *Glycine* species in the South Pacific (New Caledonia, Vanuatu, Tonga) and in Australia (Queensland including Great Barrier Reef islands, Western Australia, Northern Territory). The collecting trips were sponsored by the Rockefeller Foundation and the International Board of Plant Genetic Resources. The latter collecting trips were conducted jointly with scientists from CSIRO/Canberra. Seed of *Glycine tabacina* and *G. tomentella* were collected from plants growing on sandy or coral beaches. It seemed reasonable to assume that some of these accessions were tolerant of salt.

Two preliminary growth-chamber experiments were conducted to assess possible salt tolerance of some wild perennial *Glycine* accessions. Seeds were sown in sand culture, with nutrient solution (Hoagland #2 with micronutrients) pumped through at 3-hour intervals (Hoagland and Arnon, 1950). The plants were grown at 24 C, 10 hours light at 300 $\mu Em^{-2}s^{-1}$.

In the first experiment, synthetic seawater, equivalent to natural seawater in concentrations of chloride, sodium, magnesium and sulfur, was added to the nutrient reservoirs at the time of first seedling emergence and in three additional steps at six-day intervals, to give final concentrations equal to either 10 or 30% of that of natural seawater (Weast, 1981-1982).

The final harvest was 135 days after planting. All plants produced seeds (Table 1).

In the second experiment, either 17 or 34% synthetic seawater was imposed at once after the seedlings emerged. The cultivar 'Williams' replaced *G. cyrtoloba*. The only plants to survive the 34% synthetic seawater were *G. latifolia* and *G. tabacina*.

Table 1. Effect of two concentrations of synthetic seawater on the growth of six *Glycine* species

Species	IL no.	Seawater concentration 10%	30%
		grams[a]	
G. canescens	401	7.3	11.6
G. clandestina	425	4.2	3.5
G. cyrtoloba	545	4.1	3.1
G. latifolia	359	33.1	7.7
G. tabacina	643	4.2	1.7
G. tomentella	553	15.9	18.0

[a]Total plant dry weight, 6 plants.

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